# The role of macrophages type 2 and T-regs in immune checkpoint inhibitor related adverse events

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Abstract: Immune checkpoint inhibitory (ICI) therapy represents a novel approach in a variety of cancers, with impressive survival benefit. With ICIs, however, a new spectrum of immune related adverse events (irAE) including life threatening hypohysitis has emerged. This autopsy study aimed to investigate inflammatory cells, PD-1 and PD-L1 expression in cases of patients who developed hypophysitis and involvement of other organs. We analysed 6 patients, who were treated with ICIs and developed hypophysitis. Two received an additional MAP-kinase inhibitor, MEK-inhibitor and cytotoxic chemotherapy. Besides the pituitary gland, all investigated adrenal glands (5/5) were affected; three cases had other organs involved (liver (2/6), thyroid (2/6), lung (1/6), myocardium (1/6), colon (1/6). The inflammatory cells of involved organs were further specified and PD1 and PDL-1 expression was analyzed using immunohistochemistry. We observed that patients treated with ICIs alone showed T-cell predominant lymphocytic infiltrates, whereas patients receiving additional therapies demonstrated an increase in B- and T-lymphocytes. Surprisingly, the dominant inflammatory population was not T-cell, but type 2 macrophages. CD25 positive T-regs were sparse or absent. Our study suggests that T cell activation is only partially responsible for irAE. ICI therapy interaction with CTLA-4, PD-1 and PDL-1 in type 2 macrophages appears to result in disturbance of their control. Furthermore, depletion of T-regs seems to contribute significantly. Our findings with simultaneous pituitary and adrenal gland involvement underlines the systemic involvement as well as the importance of monitoring cortisol levels to avoid potentially life threatening hypocortisolism.

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# The role of macrophages type 2 and T-regs in immune checkpoint inhibitor related adverse events

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ABSTRACT

Immune checkpoint inhibitory (ICI) therapy represents a novel approach in a variety of cancers, with impressive survival benefit. With ICIs, however, a new spectrum of immune related adverse events (irAE) including life threatening hypohysitis has emerged. This autopsy study aimed to investigate inflammatory cells, PD-1 and PD-L1 expression in cases of patients who developed hypophysitis and involvement of other organs.

We analysed 6 patients, who were treated with ICIs and developed hypophysitis. Two received an additional MAP-kinase inhibitor, MEK-inhibitor and cytotoxic chemotherapy. Besides the pituitary gland, all investigated adrenal glands (5/5) were affected; three cases had other organs involved (liver (2/6), thyroid (2/6), lung (1/6), myocardium (1/6), colon (1/6). The inflammatory cells of involved organs were further specified and PD1 and PDL-1 expression was analyzed using immunohistochemistry. We observed that patients treated with ICIs alone showed T-cell predominant lymphocytic infiltrates, whereas patients receiving additional therapies demonstrated an increase in B- and T-lymphocytes. Surprisingly, the dominant inflammatory population was not T-cell, but type 2 macrophages. CD25 positive T-regs were sparse or absent.

Our study suggests that T cell activation is only partially responsible for irAE. ICI therapy interaction with CTLA-4, PD-1 and PDL-1 in type 2 macrophages appears to result in disturbance of their control. Furthermore, depletion of T-regs seems to contribute significantly. Our findings with simultaneous pituitary and adrenal gland involvement underlines the systemic involvement as well as the importance of monitoring cortisol levels to avoid potentially life threatening hypocortisolism.

## 1. Introduction

Treatment with immune checkpoint inhibitors (ICIs) has revolutionized the approach to cancer therapy. In contrast to previous therapies, which are directly toxic to cancer cells, ICIs activate the patient's immune system by inhibiting T-cell inactivation (Buchbinder and Hodi, 2015). The tremendous survival benefit of ICIs as first line treatment was first shown for metastatic melanoma (Larkin et al., 2015a).

Currently, monoclonal antibodies against two different pathways of T-cell inactivation are FDA and EMA approved. One medication class comprising Ipilimumab and Tremelimumab inhibits the cytotoxic T-cell lymphocyte antigen-4 (CTLA-4) pathway. Another class, which includes Nivolumab and Pembrolizumab, inhibits the program death-1 receptor (PD-1) or its ligand (PD-L1) with Durvalumab and Atezolizumab (Robert et al., 2015; Borghaei et al., 2015).

ICI therapy has demonstrated impressive results in a variety of solid tumors and haematologic malignancies (Larkin et al., 2015b; Motzer et al., 2015; Brahmer et al., 2015; Rosenberg et al., 2016; Reck et al., 2016; Kaufman et al., 2016; Ferris et al., 2018; Jelinek et al., 2017). At present, published data underline the long-time survival benefit in melanoma patients, who have undergone combination therapy simultaneously targeting CTLA-4 and PD-1 pathways (Larkin et al., 2019,

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2015b).

However, a novel spectrum of autoimmune–inflammatory toxicities has emerged with ICI therapy, which differ from those classically encountered with chemotherapy or other forms of immunotherapy (Abdel-Wahab et al., 2016; De Velasco et al., 2017).

The severity and frequency of immune related adverse events (irAEs) are dose dependent (Kaehler et al., 2010). Hypophysitis has emerged as a potentially life threatening adverse event, carries a variable incidence (0–17 %). Prior to therapies with ICIs, hypophysitis was an exceptionally rare adverse event in patients treated with interferon for viral hepatitis (Caturegli et al., 2005). The pathogenic mechanism of these new adverse events is postulated to be immune related (Sanderson et al., 2005; Iwama et al., 2014).

In the present study, we have analysed the histopathology of irAEs in six autopsy-examined patients treated with Ipilimumab alone or in combination with Nivolumab or Pembrolizumab. In all cases, we observed involvement of more than one organ, indicating the systemic nature of irAEs. Besides the pituitary gland, all investigated adrenal glands (5/5) were affected. Other involved organs included the colon (2/6), lungs (1/6), thyroid (2/6), liver (2/6) and heart (1/6).

Based on the observation that ICIs predominantly activate T-lymphocytes, the assumption could be made that T cell infiltrates would be more prevalent in irAE. Interestingly, macrophages type 2 (M2) dominate the inflammatory infiltrates and are accompanied by T-reg depletion. These observations suggest that the interaction with CTLA-4, PD-1 and PDL-1 in M2 and T-regs contributes to irAE.

## 2. Material and methods

This autopsy study was approved by the Ethics Committee of the Canton Zurich (BASEC-Nr. PB 2017−27). The institution's autopsy records were retrospectively searched from 2019−2011 for patients with hypophysitis and metastatic melanoma, who had been treated with ICIs. Corresponding medical records were reviewed for clinical history, and autopsy findings.

In all cases, autopsy tissue was fixed in 4% buffered formalin, routinely processed and embedded in paraffin. Subsequently, 2–4 μm thick sections were stained with hematoxylin and eosin and elastica van Gieson. For additional postmortem studies, paraffin-embedded sections of pituitary, adrenal gland, thyroid, myocardium, lung, and liver were immunostained with commercially available antibodies for immune cells (Table 1). Additionally, an in vivo colon biopsy of patient 3 was investigated. Scoring of the inflammatory cells was evaluated semiquantitatively based on an arbitrary five-tiered scale from negative to 4+: 0 was recorded, if staining was completely absent or fewer than 1% of inflammatory cells were immunopositive, 1+ corresponded to 1–5 % of inflammatory cells, 2+ for 6–25 %, 3+ for 26–50 % and 4+ for over 50 % of inflammatory cells. For PD-1 and PD-L1, plasma membrane staining of any intensity was considered positive. Diffuse cytoplasmic staining in parenchymal cells was not counted. Granular positivity in macrophages of PD-L1 was regarded as positive. PD-1 and PD-L1 was scored separately in parenchymal and inflammatory cells using the semiquantitative Roche algorithm for tumors: - absence, 1+ positivity in <1%, 2+ positivity in 1 %–4 %, 3+ positivity in 5 %–50 % of parenchymal cells. For inflammatory cells, 3+ corresponds to 5 %–9 % positivity and 4+ for positivity in over 9% of inflammatory cells. Appropriate positive and negative controls were used.

**Table 1**
Details of immunohistochemical antibodies.

| Antibody | Clone | Dilution | Source | Platform |
|---|---|---|---|---|
| CD3 | LN10 | 1:500 | Leica | Ventana |
| CD4 | SP35 | Ventana-Roche | Prediluted | Ventana |
| CD8 | C8/144B | DAKO A/S | 1:100 | Ventana |
| CD20 | L26 | Ventana-Roche | Prediluted | Ventana |
| CD25 | 4C9 | Cell Marque | 1:100 | Bond/Leica |
| CD68 | PG-M1 | DAKO A/S | 1:50 | Ventana |
| CD163 | 163C01 / 10D6 | NeoMarkers | 1:200 | Bond/Leica |
| PD-1 | J116 | DAKO A/S | 1:50 | Ventana |
| PDL1 263 | SP263 | Ventana-Roche | Prediluted | Ventana |
| MPO | polycloncal | NeoMarkers | 1:200 | Ventana |

## 3. Results

### 3.1. Clinics

#### 3.1.1. Patient 1

A 57-year-old female was diagnosed with nevoid melanoma of the thigh with Breslow thickness 1.5 mm. A sentinel lymph node biopsy was negative. Three years later, she developed three in-transit metastases and a single brain metastasis. Therapy was started with four cycles of Ipilimumab followed by postoperative radiotherapy with 30 gy (Table 2).The patient suffered a possible immunotherapy related skin rash and thyroiditis. MRI demonstrated no signs of hypophysitis. Two weeks before death, the patient received a single infusion of Pembrolizumab. Post mortem ACTH and cortisol values were extremely low with 15 ng/l and 4 nmol/l, respectively (Table 3).

#### 3.1.2. Patient 2

A 81 year-old female was diagnosed with uveal melanoma and underwent local proton beam therapy. Three years later, she developed multiple liver metastases and was treated with selective intra-arterial radiotherapy followed by Ipilimumab and Nivolumab (Table 2). Five weeks after ICI medication, she became comatose and died.

#### 3.1.3. Patient 3

A 64-year-old male was diagnosed with melanoma of the left chest, SSM-type, Breslow thickness 1.5 mm Clark level IV with a single axillary lymph node metastasis. Three years after initial diagnosis, he developed a single brain metastasis. Postoperatively, the patient received radiotherapy and three cycles of Ipilimumab (Table 2). After the third cycle, he developed immune therapy-associated, histologically confirmed colitis and MRI evidence of hypophysitis. Prednisone, 50 mg per day, was started and continued for 5 weeks. One month after prednisone therapy was discontinued, the patient was hospitalized with mental status changes. The ACTH level was with 15 ng/l low (no: >46 ng/l) and

**Table 2**
Therapy.

| | Medication | FM | LM | RT |
|---|---|---|---|---|
| 1 | Ipilimumab | 24 | 16 | Brain |
| | Nivolumab | 1 | | 26 |
| 2 | Ipilimumab + Nivolumab | 5 | 1 | PB 36 |
| | | | SIRT 7 | |
| 3 | Ipilimumab | 23 | 9 | Brain 27 |
| 4 | Ipilimumab | 7 | 4 | LN 28 |
| 5 | Ipilimumab | 56 | 53 | Brain 8 |
| | Pembrolizumab | 52 | 40 | |
| | Tafinar + Mekinist | 40 | 36 | |
| | Zellboraf + Cotellic | 32 | 2 | |
| | Temodal | 2 | | |
| 6 | Ipilimumab | 109 | 101 | LN 9 |
| | Dabrafenib+ Trametinnib+ Spartalizumab | 48 | 17 | |
| | Vindesine+ Cisplatin | 16 | 14 | |
| | Encorafenib+ Binimetinib | 7 | | |

FM First medication before death in weeks; LM Last medication before death in weeks; RT Radiotherapy before death in weeks; PB Proton beam; LN Lymph nodes; SIRT selective intraarterial radiotherapy before death in weeks.

**Table 3**
Autopsy findings and immune related adverse events (irAE).

| ID | Residual Tumor | Hypophysitis | Adrenalitis | ACTH | Cortisol | Other irAEs |
|---|---|---|---|---|---|---|
| 1 | No | Yes | Yes | 15 ng/l | 2 nmol/l | Thyroiditis, myocarditis, pneumonitis, hepatitis |
| 2 | Liver and bone | Yes | Yes | n.a. | n.a. | No |
| 3 | No | Yes | Yes | 15 ng/l | 72 nmol/l | Colitis |
| 4 | Extensive | Yes | n.a | 1200 ng/l | 1300 nmol/l | Colitis |
| 5 | Brain | Yes | Yes | n.a. | n.a. | Thyroiditis, hepatitis |
| 6 | Extensive | Yes | n.a. | n.a. | n.a. | No |

n.a. not available.

cortisol only 72 mmol/l, (normal range: 64–327). Due to rapid general deterioration, further diagnostic steps were not undertaken and the patient died.

#### 3.1.4. Patient 4

A 63-year-old male was diagnosed with cutaneous melanoma on the neck, SSM-type, Breslow thickness 0.7 mm, Clark level IV. Neck dissection demonstrated positive lymph nodes. Five months after radiotherapy, pulmonary metastatic spread was observed and the patient received two cycles of Ipilimumab. After the second cycle, the patient was hospitalized with severe colitis and hyponatremia. ACTH (1200 ng/l) and cortisol (1300 nmol/l) levels were elevated. Cranial MRI showed no signs of hypophysitis. Two weeks after hospitalization, the patient died (Table 2 and 3).

#### 3.1.5. Patient 5

A 44-year-old female was diagnosed with anal melanoma, Breslow thickness 5 mm. Left inguinal sentinel lymph node biopsy was positive. Inguinal lymphadenectomy demonstrated a second, partially extranodal metastasis, which prompted postoperative radiotherapy. After months, local anal recurrence was detected as well as subcutaneous inguinal and presacral metastases. Ipilimumab therapy was started. After 2 cycles of Ipilimumab, inguinal tumor progression was noted and Pembrolizumab therapy was started. Subsequently, the patient developed autoimmune thyroiditis and therefore received Euthyrox. Despite one infusion of Pembrolizumab, a thoracic vertebral body metastasis developed. Therapy was switched to MEK inhibitors, including Tafinar and Mekinist, which triggered an auto-inflammatory syndrome with SIRS and MODS. Therefore, combination therapy including Zelboraf and Cotellic was started. However, cerebral metastases were detected. Whole brain irradiation and operation were performed. Five weeks before death, therapy was changed to Temodal (Table 2).

#### 3.1.6. Patient 6

A 34-year-old male was diagnosed with melanoma on the right chest, nodular type with Breslow thickness 3.4 mm and lymph node metastases. Despite 4 cycles of Ipilimumab, there was tumor progression with thoracic metastases. The patient developed irAEs with hypophysitis and colitis after 11 weeks of therapy. ACTH and cortisol levels were low with 21 ng/ml and 79 mmol/l, respectively (Table 3). Glucocorticoid therapy with 2 × 4 mg dexamethasone per day was started and then changed to prednisone. Fifteen weeks after initial therapy, he received 30 mg hydrocortisone per day. Hydrocortisone was gradually stopped after 8 weeks.

Six month later, the patient developed paraaortic lymph node metastases. Therapy with Dabrafenib, Tramatinib and Spartalizumab was started; however, progressive disease developed. Combination therapy was stopped and the patient underwent percutaneous radiotherapy with 13 × 3 Gy. In addition, he received two infusions of Anti PD-1 and LAG. The disease progressed relentlessly with evidence of lung and bone metastases. A single cycle of XGEVA was administered in combination with BRAFTOVI and MEKTOVI (Table 2). Tumor progression continued with death ensuing 31 month after initial diagnosis.

### 3.2. Autopsy findings

#### 3.2.1. Macroscopy

Patient 4 and 6 demonstrated extensive metastatic disease involving the lymph nodes, lung, liver, kidney, bones, mediastinum and heart. Patient 2 had liver and bone and patient 5 only brain metastases. No residual tumor was found in patients 1 and 3 (Table 3).

#### 3.2.2. Histopathology

All patients had inflammation of the pituitary gland. Additionally, all investigated adrenal glands (5/5) were also inflamed. In patient 1, thyroiditis, myocarditis, pneumonitis and hepatitis was seen and in patient 5, thyroiditis and hepatitis.

#### 3.2.3. Adrenal and pituitary gland of patient 1–6

Investigated adrenal glands demonstrated macrophage predominant inflammation and mild fibrosis were observed. Specifically, type 2 macrophages with co-expression of CD68 and CD163 comprised over 50 % of inflammatory cells. The infiltrate was accompanied by 26 %–50 % T-lymphocytes with equally distributed positivity for CD4 and CD8 in 2 patients, whereas one patient showed a slight CD4 and another slight CD8 predominance. With the exception of patient 5, who received additional to immune check point inhibitors kinase inhibitors, B-lymphocytes were absent or less than 5% percent. CD25 positive T-regs were found in less than 1% of all investigated adrenal glands. Few to moderate PD-L1 positivity was found in macrophages in 3 cases and in the parenchymal cells of 2 patients (Fig. 1a A–L; Table 4).

Five of 6 pituitary glands showed a M2 rich inflammation accompanied by few to moderate lymphocytes. Patient 3 showed pronounced atrophy and fibrosis. B- and T- lymphocytes were roughly equally distributed or fewer in 4 of 5 patients (Fig. 1b A–J; Fig. 1c E–H). Only patient 6, who received additional kinase inhibitors and cytotoxic chemotherapy, demonstrated a B-cell predominance over T-cells. CD25 was present in single cells in 4 of 5 patients. Parenchymal PD-L1 positivity was found in 5/5 pituitary glands. Three patients showed 5 %–50 % positivity in parenchymal cells and 2 in less than 1%. Four of 5 patients had less than 1% PD-L1 positive macrophages (Fig. 1b K and L). PD-1 was negative in parenchymal and inflammatory cells, with the exception of patient 3 with 1 %–4 % positivity of inflammatory cells. The anterior pituitary gland of patient 4 showed acute necrosis with granulocytic reaction in with macrophage rich inflammation (Fig. 1c A–D). There were only occasional CD4 or CD8 positive lymphocytes. Unfortunately, there was insufficient material to perform staining for CD163, CD20, CD25, PD-1 or PD-L1,

#### 3.2.4. Thyroid gland of patients 1 and 5

Patient 1 demonstrated a M2 predominant, destructive thyroiditis with moderate T-cell and sparse B-lymphocytic infiltrates. Many M2 and thyroid gland cells were positive for PD-L1 and few T-lymphocytes for PD-1. The thyroid of patient 5 was markedly atrophic with prominent lymphocytic inflammation accompanied by few M2. B- lymphocytes represented over 50 % of inflammatory cells. Between 5% and 9% of lymphocytes were positive for PD-1. PD-L1 was negative. CD25 positive

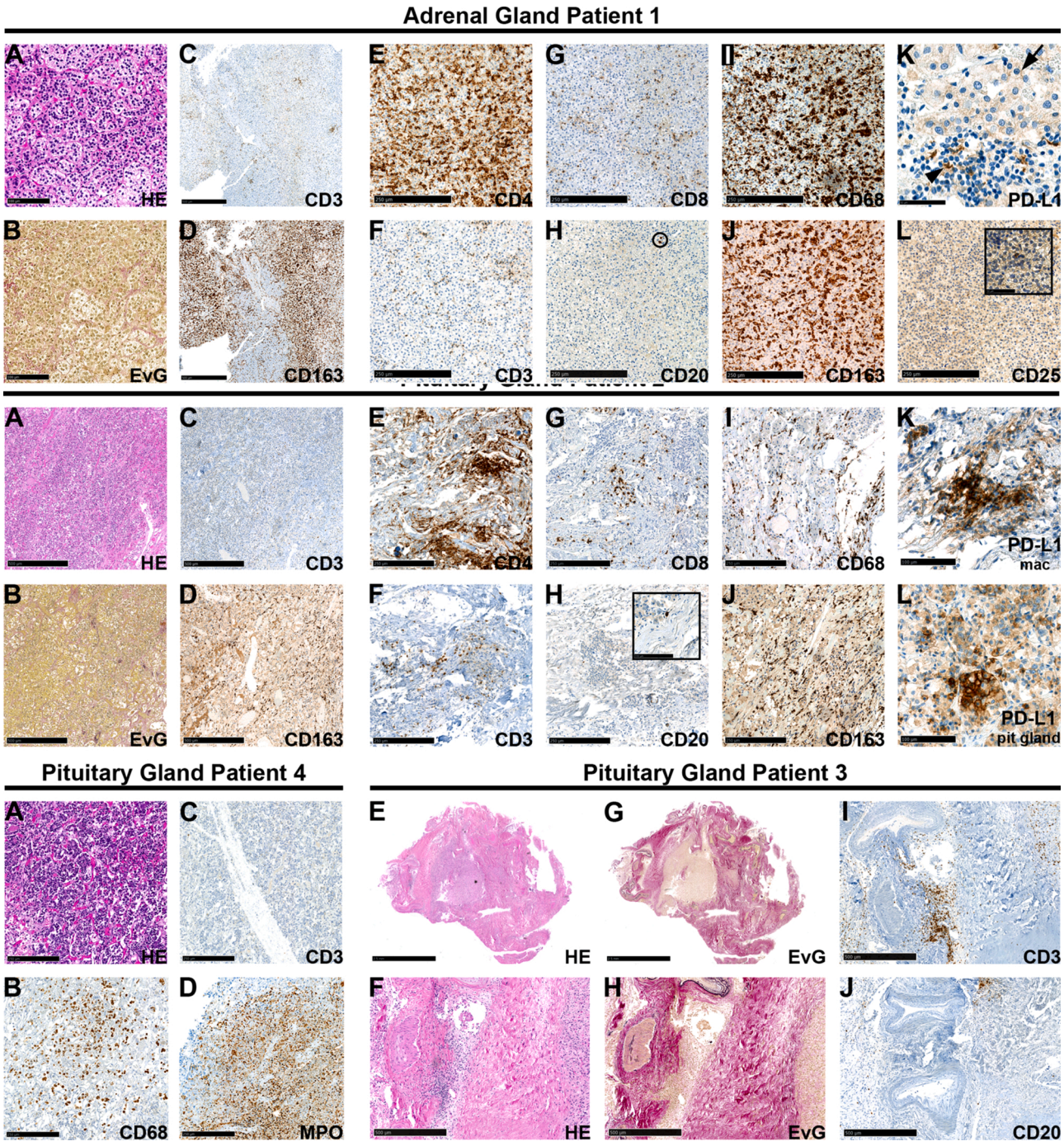


**Fig. 1.** a A-L: Adrenal gland (patient 1) with pronounced inflammation and mild fibrosis (A: Hematoxylin and eosin; B: Elastica van Gieson). Overview with moderate CD3 positive lymphocytes (C) and extensive CD163 positive macrophages type 2 (D). F and J: high-power views of C and D. E-J: Same areas with many CD4 positive cells, mostly representing macrophages with co-expression of CD68 (I) and CD163 (J). T-lymphocytic infiltrates with equal distribution of CD4 (E) and CD8 (G). Single B-lymphocytes with positivity for CD 20 (encircled, H). Only rare CD25 positive T-cells (L; inset: positive control). PDL-1 positive macrophages (arrowhead) and weak membranous positivity in parenchymal cells (arrow, K). b A-L: Pituitary gland (patient 2) with pronounced inflammation and moderate fibrosis (A: Hematoxylin and eosin; B: Elastica van Gieson). Overview with moderate CD3 positive lymphocytes (C) and extensive CD163 positive macrophages type 2 (D). F and J: high-power views of C and D. E-L: Same areas with many CD4 positive cells mostly representing macrophages with co-expression of CD68 (I) and CD163 (J). T-lymphocytic infiltrates with slight CD4 (E) predominance over CD8 (G). Absence of B-lymphocytes (inset with positive control; H). Extensive positivity for PDL-1 with predominance of anterior pituitary gland cells (L) over macrophages (K). c A-D: Patient 4 with acute necrosis of the anterior pituitary gland and myeloperoxidase positive granulocytic (D) and CD 68 positive (B) macrophage rich inflammation and few T-lymphocytes (C). E-J: Patient 3 with pronounced atrophy and fibrosis of the pituitary gland, especially in the anterior gland (E: Hematoxylin and eosin; G: Elastica van Gieson). F and H close up views of E and G. Moderate CD3 positive (I) T-lymphocytic infiltration and few CD20 positive (J) lymphocytes.

**Table 4**
Immunohistochemical analysis of inflammation, PD1 and PDL-1 expression.

| Patient | Organ | CD3* | CD4* | CD8* | CD25* | CD20* | CD68* | CD163* | PD1** | PD-L1 P/I*** |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Pit. Gl. | 2+ | 2+ | 2+ | 1+ | 2+ | 3+ | 3+ | – | 3+/1+ |
| 1 | Adr. | 3+ | 2+ | 2+ | – | – | 4+ | 4+ | – | 1+/1+ |
| 1 | Thyroid | 3+ | 2+ | 2+ | 1+ | 1+ | 4+ | 4+ | 2+ | 3+/2+ |
| 1 | Lung | 3+ | 3+ | 1+ | – | 1+ | 4+ | 4+ | – | -/3+ |
| 1 | Heart | 3+ | 2+ | 2+ | – | – | 3+ | 3+ | – | -/1+ |
| 1 | Liver | 3+ | 3+ | 1+ | – | – | 4+ | 4+ | – | -/1+ |
| 2 | Pit. gl. | 3+ | 3+ | 2+ | – | – | 3+ | 3+ | – | 3+/1+ |
| 2 | Adr. | 3+ | 2+ | 3+ | – | 1+ | 4+ | 4+ | – | 2+/3+ |
| 2 | Liver | 3+ | 2+ | 3+ | – | – | 4+ | 4+ | – | -/4+ |
| 3 | Pit. gl. | 3+ | 3+ | 2+ | – | 1+ | 4+ | 4+ | 2+ | 1+/- |
| 3 | Adr. l.s. | 3+ | 3+ | 2+ | – | 1+ | 4+ | 4+ | – | -/- |
| 3 | Adr. r.s. | 3+ | 3+ | 2+ | – | 1+ | 4+ | 4+ | – | -/1+ |
| 3 | Colon | 2+ | 2+ | 2+ | – | 1+ | 4+ | 4+ | 1+ | -/3++ |
| 4 | Pit. gl. | 1+ | 1+ | 1+ | n.a. | n.a. | 4+ | n.a. | n.a. | n.a. |
| 5 | Pit. gl. | 2+ | 2+ | 2+ | – | 2+ | 3+ | 3+ | – | 1+/1+ |
| 5 | Adr. | 2+ | 2+ | 2+ | – | – | 4+ | 4+ | – | -/- |
| 5 | Thyroid | 3+ | 3+ | 3+ | – | 4+ | 2+ | 2+ | 3+ | -/- |
| 6 | Pit. gl. | 2+ | 2+ | 2+ | – | 3+ | 3+ | 3+ | – | 3+/1+ |

Pit. gl., Pituitary gland; Adr., Adrenal gland; l.s, left side; r.s., right side; n.a., not available;
* Semi-quantitative scoring scale for inflammatory cells: - absence or positivity in <1% of inflammatory cells ; 1+ positivity in 1–5 %, 2+ positivity in 6–25 %, 3+ positivity in 26–50 % and 4+ positivity in over 50 % of inflammatory cells.
** Semi-quantitative scoring scale for PD-1: - absence, 1+ positivity in <1%, 2+ positivity in 1% and 4%, 3+++ positivity in 5 %–10 % of inflammatory cells.
*** Semi-quantitative scoring scale for PD-L1 in parenchymal (P) and inflammatory (I) cells: - absence, 1+ positivity in <1%, 2+ positivity in 1 %–4 %, 3+ positivity in 5 %–50 % of parenchymal (P) cells. For inflammatory cells (I) 3+ corresponds to a positivity in 5 %–9 % and 4+ to a positivity over 9% of inflammatory cells.

cells were sparse or absent in both glands (Table 4; Fig. 2a)

### 3.2.5. *Liver of patient 1 and 2*

In the liver of both patients, over 50 % of inflammatory cells were M2 accompanied by a moderate T-cell infiltration. In patient 1, CD4, and in patient 2, CD8 positive T-lymphocytes predominated. Only single cells were positive for CD20 or CD25. M2 were positive for PD-L1 in over 9% in patient 2 and less than 1% in patient 1 whereas liver cells were negative in both patients. Macrophages and liver cells were PD-1 negative Fig. 3b).

### 3.2.6. *Lungs of patient 1*

Focal M2- rich interstitial inflammation was observed with moderate CD4- and very few CD8-postive infiltrates. CD25 positive T-cells and

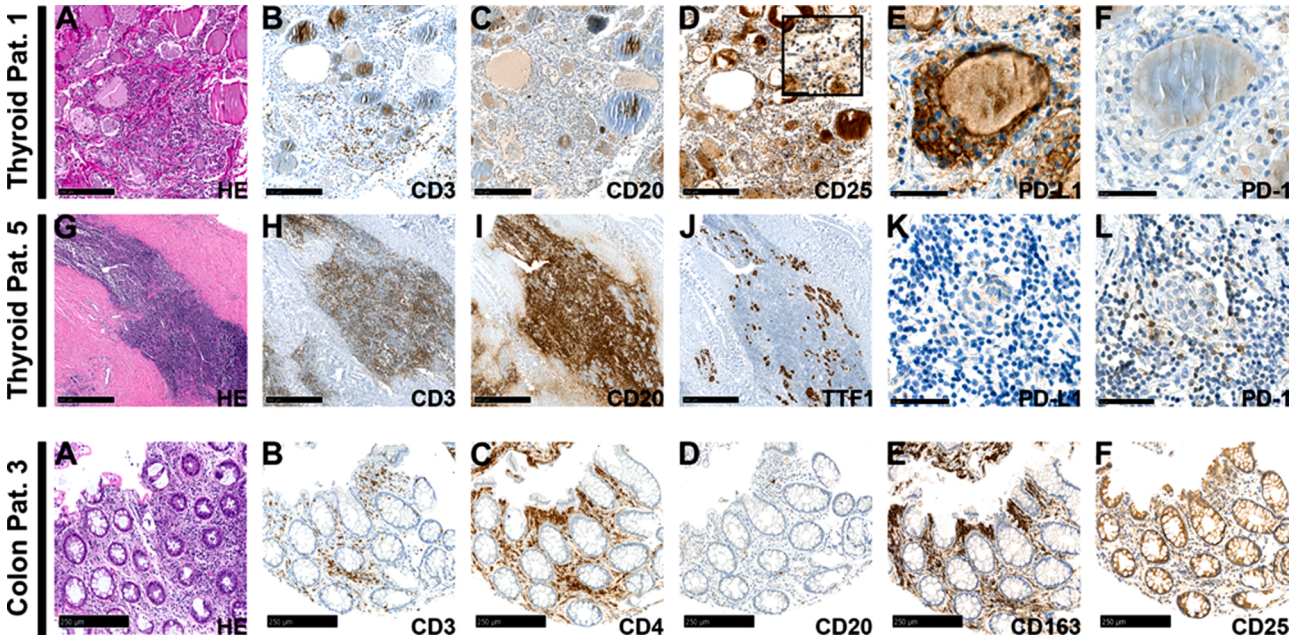


**Fig. 2.** a Comparison of thyroid gland of patient 1 (A-F) and patient 5 (G-L): Patient 1 with M2 macrophage rich lymphocytic thyroiditis. Moderate numbers of CD3 positive T-cells (B), few numbers of CD25 positive T-regs (inset high-power view; D) or CD20 positive B-cells (C). In contrast, patient 5 with atrophic heavy lymphocytic infiltrated gland, predominated by B (I) over T-cells (H). Atrophic gland illustrated with positivity for TTF-1 (J). Patient 1 with numerous plasma membranous PD-L1 positive thyroid gland cells (E), whereas patient 5 has weak diffuse cytoplasmic staining in thyroid gland cells which was considered negative and negativity in macrophages (K). Glands of both patients with PD-1 positive T-lymphocyte (F, L). b Colon biopsy of patient 2: Minor hyperplastic mucosa with elongated crypts, focal moderate active inflammation and apoptosis (A: hematoxylin-eosin). Inflammation subepithelial pronounced M2 macrophage rich with positivity for CD163 (E). Moderate lymphocytic infiltration with CD3 positive partly intraepithelial T-cells (B) and few B-cells (D). Much more CD4 positive cells (C) compared to CD3 partly representing macrophages with co-expression of CD 163. Negativity for T-regs (F) in the inflamed area.

CD20 positive B-cells were rare. M2 were moderately positive for PD-L1 and PD-1 negative. Parenchymal cells were PD-1 and PD-L1 negative (Fig. 3a).

### 3.2.7. *Myocardium of patient 1*

Moderate M2 and CD4 and CD8 positive lymphocytes were found with only rare B-lymphocytes and absence of T-regs. Less than 1% of M2 were positive for PD-L1 whereas parenchymal cells were negative. M2 and parenchymal cells were PD1 negative (Fig. 3c).

### 3.2.8. *In vivo colon biopsy of patient 3*

The biopsy demonstrated mildly hyperplastic mucosa with elongated crypts, focal moderate active inflammation and increased apoptosis. The cellular infiltrate was mostly subepithelial and consisted mainly of M2 coexpressing CD68 and CD163. There was a moderate lymphocytic infiltration with CD3 positive partly intraepithelial T-cells and no obvious difference in CD4 and CD8 expression. Only few CD20 positive B-lymphocytes were present with single cells positive for CD25. PD-L1 was moderately focal positive in M2 and negative in epithelial cells. PD-1 was negative (Fig. 2b, Table 4).

## 4. Discussion

Our autopsy study investigates 6 patients with the dreaded adverse event of immune therapy related hypophysitis. All patients received CTLA-4 antibody therapy and four received additional immunemodulatory therapy against PD-1 at outset in combination with Ipilimumab or after treatment was modified. Patients 5 and 6 differ from the others, because ICI therapy was stopped 40 or 17 weeks before death. In addition, these 2 patients received BRAF and MEK inhibitors or cytotoxic chemotherapy (Table 2).

All patients treated with ICI alone, with the exception of patient 4, demonstrated mild to moderate T-lymphocytic infiltration in irAE involved organs with an equal distribution of CD4 and CD8 or slightly increased CD4-lymphocytes. Only rare B-lymphocytes were observed (Fig. 1). In contrast, patients in whom ICI therapy had been suspended and switched to tyrosine kinase inhibitors and chemotherapy, developed significant B-lymphocytic infiltrates. These observations indicate that

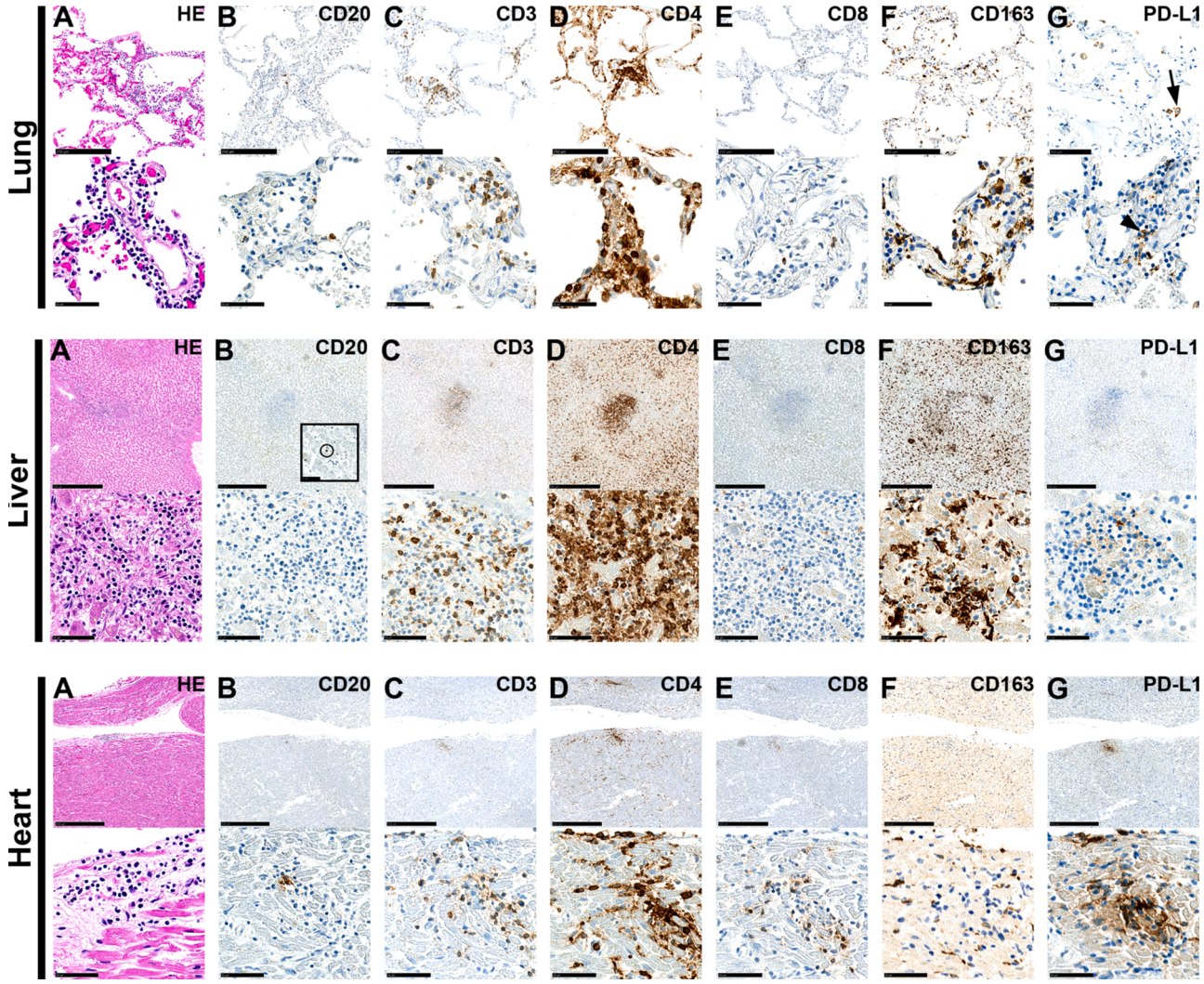


**Fig. 3.** a–c Inflammation of the lung (3a), liver (3b) and heart (3c) of patient 1 with illustration of the cellular components. Moderate T-cell predominant lymphocytic infiltrations with positivity for CD3 (C) and negativity or single cell positivity for B-lymphocytes (inset with positive control; B) in the lung septum, parenchyma of the liver and heart. T-lymphocytes mainly with positivity for CD4 (D) and rare positivity for CD8 (E). Many CD4 positive cells (D) have spindle cell morphology and co-expression of CD163 (F) representing M2 macrophages. PD-L1 positivity in macrophages with negativity in lung, liver and myocard cells (G). In the lung positive alveolar macrophages (arrow) and septal macrophages with spindle cell morphology (arrowhead).

PD-1, respectively CTLA-4 inhibiting T cell activation is partially responsible for irAEs in patients treated with ICIs. Surprisingly, the dominant inflammatory population was not T-cell, but M2 macrophages with positivity for CD68 and CD163. With the exception of the thyroid gland in patient 5, all involved organs demonstrated M2 rich inflammation. Of note, CD25 positive T-regs were sparse or absent in the organs of all patients with the exception of the thyroid and pituitary gland in patient 1 (Fig. 1 and 2). The histological features are reminiscent of the macrophage-activated syndrome (MAS) seen in systemic juvenile arthritis. This life-threatening syndrome of hyperinflammation and progressive immune-mediated organ damage is due to an overstimulated but ineffective immune response (Daver et al., 2017). However, the syndrome is based on clinical criteria, the pathophysiology is still unclear with different triggers such as infections, malignancies, autoimmune disorders and drugs. Interestingly, the syndrome has also been also described in a patient receiving Pembrolizumab (Al-Samkari et al., 2019; Hantel et al., 2018; Kalmuk et al., 2020; Sadaat and Jang, 2018).

PD-1, PD-L1 and CTLA-4 expression is found not only in lymphocytes but also in monocytes/macrophages (Gordon et al., 2017; Pistillo et al., 2003; Wang et al., 2002). Whereas the function of PD-1, PD-L1 and CTLA-4 inhibiting T cell activation is understood, there is no consensus about the function of PD-1, PD-L1 and CTLA-4 in macrophages. However, studies increasingly show an inhibitory function on the immune system.

In monocyte/T-cell co cultures, it was shown that Ipilimumab engages a subgroup of non-classical FcγRIIIA (CD16)-expressing monocytes and lyses T-regs via antibody dependent cell-mediated cytotoxicity (ADCC). Patients responding to Ipilimumab displayed significantly higher baseline peripheral non- classical monocyte counts compared with non-responder patients and had fewer T-regs (Romano et al., 2015). The protective function of T-regs against auto-immune disease in mice and human is well known (Buckner, 2010). Our observations with heavy M2 inflammation and relative reduction of T-regs support the hypothesis that irAEs are at least partly due to Treg deficiency. Importantly, the lack of T-regs may contribute to a favorable response to ICI therapy (Romano et al., 2015). However, CTLA-4 expression is also well known in T-regs, as well as their role in suppression, T cell receptor hyposignaling and anergy (Tai et al., 2012). One could therefore hypothesize a direct CTLA-4 antibody dependent, cell-mediated cytotoxicity against T-regs accompanied by a secondary M2 rich type II immune reaction. Since T-regs are sparse or absent after cessation of CTLA-4 antibody therapy, the impairment seems irreversible or perhaps other mechanisms are involved.

Another study demonstrated that in sepsis patients, peritoneal macrophages expressed high levels of PD-1 and were anergic with lower bactericidal capacity. In addition, PD-1 -/- mice with sepsis had lower mortality and a decreased bacterial burden compared with wild-type mice with sepsis (Huang et al., 2009). Studies with tumor associated macrophages (TAM) showed that PD-1 positive TAM correlate with decreased phagocytic potency against tumor cells. In addition, blockade of PD-1 or PD-L1 in vivo increases macrophage phagocytosis and reduces tumor growth. TAM are mainly M2, have higher levels of CD4 and are associated with tumor progression (Gordon et al., 2017; Sica et al., 2006). Our observation of minimal or absent PD-1 expression in CD4 positive macrophages type 2 suggests that therapy with anti-PD-1 impairs PD-1 expression and function in macrophages and might therefore be responsible for the uncontrolled immune response.

There is increasing evidence that macrophages lead to inflammation and autoimmunity due to an increased pro-inflammatory M1/anti-inflammatory M2 ratio (Smith et al., 2009; Zhu et al., 2014). Traditionally, there has been a clear dichotomy between pro-inflammatory (M1) and immunosuppressive (M2) macrophages (Fang et al., 2018). However, evidence is mounting that the situation is far more complex. Currently, three subsets of M2 (M2a, M2b, M2c) macrophages are recognized. M2b macrophages also exhibit a pro-inflammatory effect with cytokine production and Th2 activation as well as participation in type II and allergen reactions (Martinez and Gordon, 2014; Varin et al., 2010). However, in ICI therapy related inflammation there is a clear-cut predominance of a M2 destructive process, indicating a type II reaction accompanying the T-cell mediated process.

In the pituitary, CTLA-4 expression responsible for type II reaction has been reported in patients treated with anti-CTLA-4 antibody Ipilimumab (Caturegli et al., 2016; Iwama et al., 2014). We performed immunohistochemistry for CTLA-4 with the same antibody in all organs involved with irAEs. However, partly due to nuclear and non-specific immune-reactions, we were not able to evaluate the staining. Interestingly, in all cases we observed moderate to strong expression of PD-L1 in the pituitary (Table 4; Fig. 1b L). Since our patients received anti- PD-1 therapy, a direct cytotoxic reaction against PD-L1 is unlikely. However, this observation could be relevant for patients treated with anti-PD-L1.

PD-1 is expressed in hematopoietic cells (Sun et al., 2018). A reaction between PD-1 antibody and PD-1 positive M2 macrophages could account for suspected type II irAEs in patients treated with PD-1 antibody. The observed minimal or absent PD-1 expression despite heavy M2 inflammation could sustain this scenario. However, PD-1 expression was minimal or absent, even in patients 5 and 6, who received no anti-PD-1 therapy, 17 respectively 40 weeks before death. This raises the question whether PD-1 expression in macrophages is impaired for other reasons.

PD-L1 expression was observed in M2 in several different organs. (Table 4; Fig. 1a, 1b, 2a, 3a-c). A study reported that PD-L1 expression does not inhibit T-cell response but protects macrophages from destruction by T-cells, unlike PD-L1 expression on tumor cells (Sica et al., 2006). This mechanism could support our observed accumulation of M2.

Our findings with concurrent alterations in several different organs illustrate that irAEs represent a systemic disease in patients undergoing ICI therapy. Adrenalitis was observed in all four patients with hypophysitis. In two patients with available ACTH and cortisol levels, the measurements were low. A single case showed acute pituitary gland necrosis with increased serum ACTH and cortisol levels (Table 2).

Furthermore, two patients had hepatitis and thyroiditis, another pneumonitis and one myocarditis (Table 3). Of clinical importance is our observation of the dual impairment of the pituitary and adrenal gland in patients undergoing ICI therapy, which was responsible for life threating, low cortisol levels.

The strength of our study is the availability of autopsy tissue from multiple organs of six patients. The weakness of the study is that patients received different medications. Since some autopsies were analyzed in more detail retrospectively, not all adrenal glands were available.

## 5. Conclusion

Side effects caused by immunotherapy are a systemic disease. T-cell activation is only one part of the pathogenesis. Progressive M2 immune-mediated organ damage and depletion of T-regs also play an important role.

## Author contribution

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Data analysis and interpretation: Daniela Mihic-Probst, Elisabeth J Rushing

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## Ethics declarations

The patient provided a written informed consent in accordance with the Declaration of Helsinki. This prospective autopsy study was carried out with the approval of the Ethics Committee of Canton Zurich (BASEC-Nr. PB 2017−27)

## Declaration of Competing Interest

The authors report no declarations of interest.

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